Notebook[1]

iGEM 2022, University of Sheffield

March 25, 2023

[1] from https://2022.igem.wiki/sheffield/notebook

A notebook to keep track of progress

Here we have outlined all of our wet and dry lab experiments over the course of the project. Here we design a modular, plasmid-based toolkit for continuous directed evolution and a DIY bioreactor for automated continuous culture.

March

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April

To practice fundamental microbiology techniques and familiarise non-biology team members with the lab, we decided that amplifying DNA using E. coli was a good place to start. We used DNA from 2019's iGEM distribution kit and amplified them by transforming into bacterial cells. We also completed our team's registration for the Grand Jamboree.

May

Unfortunately May was exam period at the University of Sheffield and final projects were being submitted. In the meantime, team members focused on researching the shortlisted ideas before we decided on the final project in our next meeting.

June

Our first team meeting with our PI - Professor Tuck Seng Wong - was held which resulted in a slight change to the project's initial scope in order to be more achievable within the iGEM timeframe. He suggested using the **MutaT7 system** for cellular in vivo continuous directed evolution to evolve a prokaryotic 1–4 Galactosyltransferase for increased expression and lactose production. We looked into identifying a lactose biosensor to tie fitness to improved lactose production. Professor Tuck Seng Wong also suggested exploring the lac repressor while designing our genetic circuit for selection. The

Mutat7 system could be applied to other iGEM teams' parts during collaborations. We also discussed using JMP's sponsorship software to optimise VMax Express growth and expression even further. We discussed using the HiSCRIBE system as a hypermutation mechanism - to increase error rate by deleting a proofreading domain and never before published mechanism for directed evolution

We worked through setting up some E. coli overnight cultures, experiencing issues with aseptic technique and use of cells in solution rather than using plate colonies. We learnt from this however and can improve for the next overnight culturing session. Using the overnight cultures, we worked on building some E. coli growth curves by measuring incubated samples' optical density every 20 minutes over 3 hours. Ideally it would have run until the growth plateaued (considered when 3 consecutive readings show no change), but there was not enough time. However, enough data was collected to approximate a doubling time for growth.

Start of Lab Work

Week 1

Week 1 of July consisted of meetings with our PIs from engineering departments to discuss whether our ideas for the shape of the bioreactor were suitable and to further gain advice regarding the use of turbidostats. We also submitted the impact grant application and were very excited to hear the results in due course. Planning the team dynamic was a crucial part at this stage in the project; we assigned subteam leaders to give people directionality and ensure all team members were working in tandem.

We started investigating genetic parts we would like to work with. This included the MutaT7 system (Paris Saclay 2021, Alvarez, Moore), the HiSCRIBE system, and various growth-based selection mechanisms. As well as this we began designing proof of concept assays (e.g. silent ATG start codons).

On the dry lab side of things we began familiarising ourselves with various software (e.g. Fusion 360) for 3D modelling of the bioreactor. We started coding bioreactor parts and our engineers sketched first drafts of the toroidal bioreactor and ordered parts.

In the lab we completed growth curves for *V. natriegens* for media optimization.

Week 2

This week we performed lots of growth curves and eliminated candidate ingredients that negatively impacted *V. natriegens* growth to aid

media optimisation.

We got in touch with the makers of HiSCRIBE to get plasmid maps to work with, as well as emailing our sponsor JMP to get details on biochemical pathway optimization.

We also identified lots of growth slowing genes to design our selection plasmid, and sourced plasmid backbones as well as finalising the design of the MutaT7 plasmid. We decided the lac promoter we hoped to use was too leaky for our selection mechanism to work (risk of evolutionary cheaters) so decided to improve the lac promoter to contribute to part improvement. We also designed our test casset for MutaT7

We tested the Raspberry Pi with the code we had written and built a prototype of our bioreactor to model fluids and heating components. We ordered components for the stirring mechanism to test in week 3.

Week 3

We decided that a phototransistor was the best option for OD measurements. We explored the phototransistor and LED pair components to do refraction modelling to see how the refractive index of perspex would affect the rays of light from the LED - some LED pairs caused internal refraction along with the bioreactor walls. Looking into finding LEDs with narrower range and transistors that are highly sensitive. Then, we continued with modelling with LEDs and transistor pairings.

Week 4

In the lab, we finished our growth curves with e2yt as well as testing *V. natriegens* growth on sorbitol. We also mini prepped our backbones (pUC18/19). Arjun generated recipe cards for media using his design of experiments model in the JMP software to perform more growth curves to hone in on the optimal media for *V. natriegens*.

We finished designing the MutaT7 test cassette and MutaT7 fusions. Going to take a swing at ordering the sequences today in g-blocks as well as ordering anhydrotetracycline (aTc) inducer for fusion plasmid.

Chalisa did some modelling for oxygen diffusion in the bioreactor. We ordered more parts and found a perfect ceramic sealant for our bioreactor that would fix leakages, create more turbulence thus leading to better mixing of the media and also manipulate the bioreactor into a dish bottom shape. We also connected the peristaltic pump to the breadboard. Worked fine, but heated up when connected to the motor controller. Possibly short-circuited somewhere.

We also collaborated with Ohio State University. We wanted to hear their insights into how they modelled their own bioreactors virtually. Our coding member Kesler shared some useful python code for a system of ODEs that could help their team. We hope to receive a useful set of growth modelling codes from OSU for our experimental data and approximate rate constants for V. natriegens that could be substituted in.

Week 5

Media optimisation experiments continued with more media versions being made up by several lab members. It was found that Na_2HPO_4 and $MgCl_2$ forms an insoluble magnesium phosphate precipitation when both are added in media, but a work around was constructed by Arjun through swapping $MgCl_2$ for $MgSO_4$ and replacing Na_2HPO_4 for more $CaCl_2$. We failed to transform *V. natriegens* with pUC18/19, but the E. coli transformations worked. We will remake competent *V. natriegens* to see if that is the issue. We fixed our main issue with our media that kept precipitating by removing $NaPO_4$.

For the bioreactor we: gathered the equipment; connected the L239D to the breadboard; connected the peristaltic pump, motor and Raspberry Pi Pico; established micropython code and hardware set up; filled the prototype with water and pump water in a cycle and measured flow rate. The pump speed and flow rate was suspiciously low- we realised something was short of circuit due to the power supply overheating quickly. We also identified a small leakage in the bioreactor prototype which we needed to prioritise by sealing with more acrylic cement. We also built a second prototype to test stirring using magnetic balls and magnetic rods.

Week 6

This week we recorded our promotional video! :)

Our *V. natriegens* transformations are still not working well. We got a few colonies to miniprep and sequence.

We also miniprepped a bunch of plasmid backbones:

- pET21a - AmpR
- pBBRBB - KanR
- pBBRBB - SmR
- pET28a - KanR
- pBR52b - AmpR

- pACYDuet - CmR

For the bioreactor, we worked on the peristaltic pump. Solder wires onto the peristaltic pump to secure it, divert the wire from the power supply (so we can supply the breadboard with 5V and 12V) and follow the hardware setup and code. We measured the **volumetric flow rate (= 60mL/min)** and checked voltage across different connections using a multimeter (10V between motor connections, 12V supplying L293D showing power loss within the circuit). Moving forward we will make sure **Raspberry Pi Pico** is powered by 5V (not 12V), there are no unwanted wires disconnected from the breadboard but this may accidentally short the circuit, switch off the circuit before changing any connections. Resistance caused around 40% loss in power supply to the motor, so the voltage supplied is only 10V - why the volumetric flow rate is only 60mL/min. While checking the voltage, current and power between different points in the circuit, the Raspberry Pi blew up (most likely accidentally connected the Raspberry Pi to 12V instead of 5V). After this we built our third bioreactor prototype and it was concluded that a fourth prototype needed to be created- one with refined lids and deeper/wider base etchings.

Week 7

This week we acquired CRISPRi plasmid from our university department. We also completed the travel Risk assessment for Paris and Munster. We designed the layout of our poster and presentation and planned our promotional video.

In the lab, we planned an experiment for baseline activity of GalT and a cloning strategy for RNA attenuator as well as planning out final media optimisation experiments (Arjun)

We then recorded all of the bioreactor's prototype journey so far!

Week 8

We decided controlling Pump Power with Potentiometer was a good idea to ensure there was a way to turn the pump off completely and we tried to figure out quantitative value for speed for the Servo Motor. For the OD system, the original code reported the OD as transmittance rather than absorbance so we modified it to 100-light so that it measures absorbance instead. We addressed the outflow port issue, implemented a new downward tube to overcome surface tension issues outlined in the Engineering Success page. We finalised the design for the temperature sensor on the screen of the bioreactor.

Some of our team attended the European meetup in Muenster, Germany where we gained and received feedback about our posters,

presentation delivery and overall project! We also made

Week 9

More bioreactor testing, more growth curves (but now done in baffled flasks). We used HiFi assembly to generate all of our constructs: the MutaT7 test cassette, MutaT7 base editor fusions, growth slowing genes/ SDH under the control of a lac promoter and our part improvement (improved Lac promoter controlling the expression of GFP so we can compare the strength of the original promoter, to our improved promoter). Upon inducing the lac promoters we found no significant expression - maybe our IPTG induction is not working. We sent off our constructs for sequencing to confirm we constructed the correct things. Restriction digests confirmed our assemblies were correct.

Week 10

Wrapped up experiments and moved the team's complete focus to the Wiki page. Our team collaborated with Women in Engineering (WiE) Society for an outreach session to Arbourthorne Community Primary School. The session consisted of modelling DNA using plastic beads that were transformed into keychains for the children to take home.